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Sporothrix brasiliensis, *S. globosa*, and *S. mexicana*, Three New *Sporothrix* Species of Clinical Interest[▽]

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***Sporothrix schenckii* is the species responsible for sporotrichosis, a fungal infection caused by the traumatic implantation of this dimorphic fungus. Recent molecular studies have demonstrated that this species constitutes a complex of numerous phylogenetic species. Since the delineation of such species could be of extreme importance from a clinical point of view, we have studied a total of 127 isolates, most of which were received as *S. schenckii*, including the available type strains of species currently considered synonyms, and also some close morphological species. We have phenotypically characterized all these isolates using different culture media, growth rates at different temperatures, and numerous nutritional tests and compared their calmodulin gene sequences. The molecular analysis revealed that *Sporothrix albicans*, *S. inflata*, and *S. schenckii* var. *luriei* are species that are clearly different from *S. schenckii*. The combination of these phenetic and genetic approaches allowed us to propose the new species *Sporothrix brasiliensis*, *S. globosa*, and *S. mexicana*. The key phenotypic features for recognizing these species are the morphology of the sessile pigmented conidia, growth at 30, 35, and 37°C, and the assimilation of sucrose, raffinose, and ribitol.**

Sporothrix schenckii is a dimorphic fungus causing sporotrichosis, a severe infection usually acquired by the traumatic inoculation of colonized materials or by inhalation of spores through the respiratory tract (3, 6). Cutaneous lymphatic disease is the most common clinical manifestation, although other types of disease including disseminated infection are also produced. Sporotrichosis has a worldwide distribution, especially in tropical and subtropical areas. The natural habitat of *S. schenckii* is soil and plants. The teleomorph of this fungus has not yet been discovered, although a close genetic relationship between *S. schenckii* and the ascomycetous genus *Ophiostoma* has been demonstrated (2, 3). Contrary to previously reported suggestions, *Ophiostoma stenoceras* appears (5) not to be the teleomorph (4, 8, 27, 30). In recent years, numerous molecular studies involving *S. schenckii* have been carried out (13, 15, 22, 23, 25, 32, 34) and have clearly demonstrated the existence of several groups that are genetically different. In a recent multilocus study, we investigated the population structure of *S. schenckii* and showed the existence of at least six putative phylogenetic species prevalent in different geographical regions (20). In several in vitro antifungal susceptibility studies of clinical isolates of *S. schenckii*, a wide range of susceptibility to different drugs has been demonstrated (16, 21, 31). This suggests that these isolates could represent different species. If true, knowledge of their various responses to antifungal agents would be critical for appropriate patient management.

The aim of the present study was to phenotypically characterize the different phylogenetic species of the *S. schenckii* complex in order to find key morphological and/or physiological features that would allow their recognition in the clinical laboratory. Only their reliable identification will allow us to study their epidemiology and to determine if different clinical patterns are associated with each of these species. Numerous additional isolates were included in the study in order to increase the robustness of the isolate sets representing the different species detected within the complex. These isolates were assigned to different lineages on the basis of their calmodulin sequences, the most phylogenetically informative locus found in our previous study (20).

MATERIALS AND METHODS

Fungal isolates. One hundred twenty-seven isolates were included in the study (Table 1). Isolates consisted of strains that were morphologically identified as being *S. schenckii* (mainly from clinical origin), the type strain of *S. schenckii* var. *luriei*, the available type strains of species currently considered synonyms of *S. schenckii* (*Sporothrix albicans*, *Sporotrichum tropicale*, and *Dolichoascus schenckii*), and the type strain of *Sporothrix inflata*, the morphologically closest species to *S. schenckii*. Isolates were stored on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) at 4 to 7°C and in slant cultures submerged in mineral oil at room temperature.

DNA extraction, amplification, and sequencing. The procedures for DNA extraction, amplification, sequencing, and phylogeny analysis of the nuclear calmodulin (CAL) gene were described previously by Marimon et al. (20). The primers used were CL1 and CL2A (24). The phylogenetic analysis was performed by using PAUP*, version 4.0b10 (28). Briefly, the most parsimonious trees were obtained after 100 heuristic searches with random sequence addition and tree bisection-reconnection branch-swapping algorithms, collapsing zero-length branches and saving all minimal-length trees (MulTrees).

Morphological studies. In order to study macroscopic features and sporulation (5, 8, 22), all the isolates were subcultured on PDA, cornmeal agar (CMA) (30 g corn, 15 g agar, 1 liter tap water), and oatmeal agar (30 g oat flakes, 1 g $MgSO_4$, 1.5 g KH_2PO_4, 15 g agar, 1 liter tap water) and incubated at 30°C in the dark. The microscopic features were determined primarily from slide cultures made on CMA after 10 to 12 days of incubation at 30°C. Coverslips were mounted in lactic

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TABLE 1. Fungal species, source code, geographical origin, and GenBank/EMBL/DDBJ accession numbers for the isolates included in the study[c]

Species	Received as species:	Isolate	Source	GenBank/EMBL/DDBJ accession no. (CAL)
S. albicans	*S. schenckii*	CBS 302.73^T	Environmental, soil, United Kingdom	AM398396[a]
S. albicans	*S. albicans*	CBS 111110	*Zootermopsis nevadensis*, Germany	AM398382[a]
S. brasiliensis	*S. schenckii*	CBS 120339^T (IPEC 16390)	Clinical, Brazil	AM116899
S. brasiliensis	*S. schenckii*	IPEC 15572	Clinical, Brazil	AM116886
S. brasiliensis	*S. schenckii*	IPEC 16042	Clinical, Brazil	AM116885
S. brasiliensis	*S. schenckii*	IPEC 16243	Clinical, Brazil	AM116877
S. brasiliensis	*S. schenckii*	IPEC 16456	Clinical, Brazil	AM116897
S. brasiliensis	*S. schenckii*	IPEC 16503	Clinical, Brazil	AM116875
S. brasiliensis	*S. schenckii*	IPEC 16550	Clinical, Brazil	AM116892
S. brasiliensis	*S. schenckii*	IPEC 16864	Clinical, Brazil	AM116889
S. brasiliensis	*S. schenckii*	IPEC 16919	Clinical, Brazil	AM116898
S. brasiliensis	*S. schenckii*	IPEC 17307	Clinical, Brazil	AM116896
S. brasiliensis	*S. schenckii*	IPEC 17331	Clinical, Brazil	AM116880
S. brasiliensis	*S. schenckii*	IPEC 17521	Clinical, Brazil	AM116874
S. brasiliensis	*S. schenckii*	IPEC 17585	Clinical, Brazil	AM116887
S. brasiliensis	*S. schenckii*	IPEC 17608	Clinical, Brazil	AM116890
S. brasiliensis	*S. schenckii*	IPEC 17692	Clinical, Brazil	AM159127
S. brasiliensis	*S. schenckii*	IPEC 17786	Clinical, Brazil	AM116884
S. brasiliensis	*S. schenckii*	IPEC 17920	Clinical, Brazil	AM116888
S. brasiliensis	*S. schenckii*	IPEC 17943	Clinical, Brazil	AM116878
S. brasiliensis	*S. schenckii*	IPEC 22468	Clinical, Brazil	AM116882
S. brasiliensis	*S. schenckii*	IPEC 22493.1	Clinical, Brazil	AM116894
S. brasiliensis	*S. schenckii*	IPEC 22493.2	Clinical, Brazil	AM116883
S. brasiliensis	*S. schenckii*	IPEC 22496.4	Clinical, Brazil	AM116895
S. brasiliensis	*S. schenckii*	IPEC 22496.5	Clinical, Brazil	AM116879
S. brasiliensis	*S. schenckii*	IPEC 22543.2	Clinical, Brazil	AM116881
S. brasiliensis	*S. schenckii*	IPEC 22582	Clinical, Brazil	AM116891
S. brasiliensis	*S. schenckii*	IPEC 22593	Clinical, Brazil	AM116893
S. brasiliensis	*S. schenckii*	FMR 8337	Environmental, domiciliary dust, Brazil	AM116876
S. brasiliensis	*S. schenckii*	FMR 9034	Clinical, Brazil	AM261688
S. brasiliensis	*S. schenckii*	FMR 9035	Clinical, Brazil	AM261689
S. globosa	*S. schenckii*	CBS 120340^T (FMR 8600)	Clinical, Spain	AM116908
S. globosa	*Sporotrichum tropicale*[b]	CBS 292.55^T	Clinical, United Kingdom	AM490354[a]
S. globosa	*S. schenckii*	FMR 8594	Clinical, Spain	AM116906
S. globosa	*S. schenckii*	FMR 8595	Clinical, Spain	AM116905
S. globosa	*S. schenckii*	FMR 8596	Clinical, Spain	AM116902
S. globosa	*S. schenckii*	FMR 8597	Clinical, Spain	AM116907
S. globosa	*S. schenckii*	FMR 8598	Clinical, Spain	AM116903
S. globosa	*S. schenckii*	FMR 8601	Clinical, Spain	AM116901
S. globosa	*S. schenckii*	FMR 8602	Clinical, Spain	AM116909
S. globosa	*S. schenckii*	FMR 9020	Clinical, Japan	AM398994[a]
S. globosa	*S. schenckii*	FMR 9021	Clinical, Japan	AM398993[a]
S. globosa	*S. schenckii*	FMR 9022	Clinical, Japan	AM398995[a]
S. globosa	*S. schenckii*	FMR 9023	Clinical, Japan	AM399016[a]
S. globosa	*S. schenckii*	KMU 4208	Environmental, wheat, China	AM399002[a]
S. globosa	*S. schenckii*	KMU 4214	Environmental, reed, China	AM399003[a]
S. globosa	*S. schenckii*	KMU 4200	Environmental, reed, China	AM399004[a]
S. globosa	*S. schenckii*	KMU 4210	Environmental, soil, China	AM399005[a]
S. globosa	*S. schenckii*	KMU 4116	Environmental, China	AM399019[a]
S. globosa	*S. schenckii*	IHEM 4178	Clinical, Italy	AM399018[a]
S. globosa	*S. schenckii*	UTHSC 04-1485	Clinical, United States	AM399015[a]
S. globosa	*S. schenckii*	UTHSC 05-127	Clinical, United States	AM398992[a]
S. globosa	*S. schenckii*	UTHSC 99-625	Clinical, United States	AM398982[a]
S. globosa	*S. schenckii*	MCCL 220029	Clinical, India	AM490358[a]
S. globosa	*S. schenckii*	MCCL 220087	Clinical, India	AM490359[a]
S. globosa	*S. schenckii*	MCCL 220038	Clinical, India	AM490360[a]
S. globosa	*S. schenckii*	MCCL 220045	Clinical, India	AM490361[a]
S. globosa	*S. schenckii*	MCCL 220082	Clinical, India	AM490362[a]
S. globosa	*S. schenckii*	MCCL 220084	Clinical, India	AM490363[a]
S. globosa	*S. schenckii*	MCCL 220040	Clinical, India	AM490348[a]
S. globosa	*S. schenckii*	MCCL 220011	Clinical, India	AM490349[a]
S. globosa	*S. schenckii*	MCCL 220030	Clinical, India	AM490350[a]
S. globosa	*S. schenckii*	MCCL 220049	Clinical, India	AM490351[a]
S. globosa	*S. schenckii*	MCCL 220010	Clinical, India	AM490352[a]
S. globosa	*S. schenckii*	MCCL 220085	Clinical, India	AM490353[a]
S. globosa	*S. schenckii*	NBRC 5984	NK	AM116900
S. globosa	*S. schenckii*	NBRC 6072	NK	AM116904

Continued on following page

TABLE 1—*Continued*

Species	Received as species:	Isolate	Source	GenBank/EMBL/DDBJ accession no. (CAL)
S. inflata	*S. inflata*	CBS 239.68^T	Environmental, wheat field, Germany	
S. schenckii	*S. schenckii*	CBS 359.36^T	Clinical, United States	AM117437
S. schenckii	*Dolichoascus schenckii*[b]	CBS 938.72^T	Clinical, France	AM490340[a]
S. schenckii	*S. schenckii*	NBRC 8158	NK	AM117438
S. schenckii	*S. schenckii*	FMR 8604	Clinical, Peru	AM117429
S. schenckii	*S. schenckii*	FMR 8605	Clinical, Peru	AM117442
S. schenckii	*S. schenckii*	FMR 8606	Clinical, Peru	AM117431
S. schenckii	*S. schenckii*	FMR 8607	Clinical, Peru	AM117428
S. schenckii	*S. schenckii*	FMR 8608	Clinical, Peru	AM117441
S. schenckii	*S. schenckii*	FMR 8609	Clinical, Peru	AM117439
S. schenckii	*S. schenckii*	FMR 8677	Clinical, Argentina	AM117436
S. schenckii	*S. schenckii*	FMR 8678	Clinical, Argentina	AM117446
S. schenckii	*S. schenckii*	FMR 8679	Clinical, Argentina	AM117445
S. schenckii	*S. schenckii*	FMR 8716	Clinical, Peru	AM399006[a]
S. schenckii	*S. schenckii*	FMR 8717	Clinical, Peru	AM399017[a]
S. schenckii	*S. schenckii*	FMR 9051	Clinical, Venezuela	AM490342[a]
S. schenckii	*S. schenckii*	FMR 9052	Clinical, Venezuela	AM490341[a]
S. schenckii	*S. schenckii*	FMR 9054	Clinical, Venezuela	AM490343[a]
S. schenckii	*S. schenckii*	FMR 9055	Clinical, Venezuela	AM490344[a]
S. schenckii	*S. schenckii*	FMR 9113	Clinical, Venezuela	AM490345[a]
S. schenckii	*S. schenckii*	FMR 9114	Clinical, Venezuela	AM490346[a]
S. schenckii	*S. schenckii*	FMR 9115	Clinical, Venezuela	AM490347[a]
S. schenckii	*S. schenckii*	FMR 9275	Clinical, Venezuela	AM490355[a]
S. schenckii	*S. schenckii*	FMR 9276	Clinical, Venezuela	AM490356[a]
S. schenckii	*S. schenckii*	FMR 9277	Clinical, Venezuela	AM490357[a]
S. schenckii	*S. schenckii*	FMR 9278	Clinical, Venezuela	AM490337[a]
S. schenckii	*S. schenckii*	FMR 9279	Clinical, Venezuela	AM490338[a]
S. schenckii	*S. schenckii*	FMR 9280	Clinical, Venezuela	AM490339[a]
S. schenckii	*S. schenckii*	IHEM 3774	Clinical, Colombia	AM117447
S. schenckii	*S. schenckii*	IHEM 3787	NK, South Africa	AM117435
S. schenckii	*S. schenckii*	IHEM 15477	Clinical, Bolivia	AM117444
S. schenckii	*S. schenckii*	IHEM 15486	Clinical, Peru	AM117432
S. schenckii	*S. schenckii*	IHEM 15489	Clinical, Peru	AM117430
S. schenckii	*S. schenckii*	IHEM 15499	Clinical, Peru	AM117434
S. schenckii	*S. schenckii*	IHEM 15502	Clinical, Peru	AM117427
S. schenckii	*S. schenckii*	IHEM 15503	Clinical, Peru	AM117433
S. schenckii	*S. schenckii*	IHEM 15508	Clinical, Peru	AM117443
S. schenckii	*S. schenckii*	IHEM 15511	Clinical, Peru	AM117440
S. schenckii	*S. schenckii*	UTHSC 05-2843	Clinical, United States	AM399012[a]
S. schenckii	*S. schenckii*	UTHSC 05-802	Clinical, United States	AM399008[a]
S. schenckii	*S. schenckii*	UTHSC 04-2235	Clinical, United States	AM398984[a]
S. schenckii	*S. schenckii*	UTHSC 04-1718	Clinical, United States	AM398990[a]
S. schenckii	*S. schenckii*	UTHSC 04-1064	Clinical, United States	AM399014[a]
S. schenckii	*S. schenckii*	UTHSC 04-1012	Clinical, United States	AM399020[a]
S. schenckii	*S. schenckii*	UTHSC 04-771	Clinical, United States	AM398985[a]
S. schenckii	*S. schenckii*	UTHSC 04-797	Clinical, United States	AM399013[a]
S. schenckii	*S. schenckii*	UTHSC 03-3124	Clinical, United States	AM398996[a]
S. schenckii	*S. schenckii*	UTHSC 03-1684	Clinical, United States	AM398989[a]
S. schenckii	*S. schenckii*	UTHSC 03-1627	Clinical, United States	AM398987[a]
S. schenckii	*S. schenckii*	UTHSC 03-823	Clinical, United States	AM398991[a]
S. schenckii	*S. schenckii*	UTHSC 02-2723	Clinical, United States	AM399007[a]
S. schenckii	*S. schenckii*	UTHSC 02-510	Clinical, United States	AM398986[a]
S. schenckii	*S. schenckii*	UTHSC 01-2137	Clinical, United States	AM398983[a]
S. schenckii	*S. schenckii*	UTHSC 00-1734	Clinical, United States	AM398988[a]
S. schenckii	*S. schenckii*	UTHSC 00-1488	Clinical, United States	AM399009[a]
S. schenckii	*S. schenckii*	UTHSC 00-603	Clinical, United States	AM399010[a]
S. schenckii	*S. schenckii*	UTHSC 99-173	Clinical, United States	AM399011[a]
S. schenckii var. *luriei*	*S. schenckii* var. *luriei*	CBS 937.72^T	Clinical, South Africa	
S. mexicana	*S. schenckii*	CBS 120341^T	Environmental, soil rose tree, Mexico	AM398393[a]
S. mexicana	*S. schenckii*	CBS 120342	Environmental, carnation leaves, Mexico	AM398392[a]

[a] Sequences newly generated in this study.
[b] Invalidly published species.
[c] Abbreviations: IPEC, Instituto de Pesquisa Clínica Evandro Chagas, Fiocruz, Brazil; FMR, Facultat de Medicina i Cieńcies de la Salut, Reus, Spain; IHEM, BCCM/IHEM Biomedical Fungi and Yeasts Collection, Belgium; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; NBRC, Biological Resource Center, Chiba, Japan; KMU, Kanazawa Medical University, Ishikawa, Japan; UTHSC, Fungus Testing Laboratory, University of Texas Health Science Center; MCCL, Mycology Culture Collection Laboratory, Postgraduate Institute of Medical Education and Research, Chandigarh, India; NK, not known; ^{T}, type strain.

acid and examined under a light microscope (Leitz Dialux 20). At least 25 measurements were recorded as the maximum and minimum values for each type of structure.

Physiologic studies. The growth rate at various temperatures (20, 25, 30, 35, 37, and 40°C) of all the isolates included in the study was determined on PDA. The petri dishes were centrally inoculated with pieces of the fungus that were approximately 1 mm in diameter, placed upside down. The colony diameters (in millimeters) were measured after 14 and 21 days of incubation. The mean of the diameters was determined to detect differences among isolates.

Assimilation of 35 carbon and seven nitrogen sources was tested in liquid medium according to methods described previously by Yarrow (33). The tests were done in 96-well microplates, with each column containing a standard 150-μl amount of liquid nitrogen base medium (Becton Dickinson Co., Sparks, MD) or carbon base medium (Becton Dickinson Co.), with one test substrate, except those for the negative controls, which had only the base medium, and those for the positive controls, which contained glucose. An inoculum of 50 μl was added to each well of the microdilution trays. The inocula were adjusted to an optical density that ranged from 0.21 to 0.29, which corresponded to a final inoculum in the microplate of 2×10^5 to 2×10^6 CFU/ml. The viability of the conidia was verified by plating 100 μl of serial dilutions of each inoculum onto PDA. Microplates were read after 5 and 10 days of incubation at 25°C.

The presence of urease was determined after incubation on Christensen's urea agar slants at 25°C for 8 days. Urease production was noted by the development of a pink color. Acid production was tested on chalk agar (50 g glucose, 5 g $CaCO_3$, 5 g yeast extract, 20 g agar, 1 liter demineralized water) at 25°C, with cultures examined regularly for up to 4 weeks for clearing of the medium around the streaks (33). Gelatin liquefaction was tested on a medium composed of 100 g gelatin, 5 g glucose, 6.7 g nitrogen base medium, and 1 liter of demineralized water and incubated at 25°C for 60 days (33). After incubation, tubes were then refrigerated at 4°C for 1 h to check gelatin hydrolysis. Tolerance to NaCl, $MgCl_2$, and cycloheximide was tested as described above for the assimilation of carbon sources but in liquid nitrogen base medium with 5% glucose (7). Final concentrations were 0.1%, 0.25%, and 1% and 2%, 5%, and 10% for cycloheximide and NaCl and $MgCl_2$, respectively. The results were both read after 7 and 14 days of incubation at 25°C. The formation of extracellular polysaccharide was performed on agar medium in petri dishes, incubated at 25°C for 2 to 3 weeks, and then flooded with diluted Lugol's iodine and examined for the formation of a blue-green color (33).

Testing of the ability of isolates to convert to the yeast phase was performed according to procedures described previously by Ghosh et al. (9). Briefly, mycelial cultures grown on PDA were subcultured on brain heart infusion agar with 5% defibrinated sheep blood at 37°C for 6 to 9 days. Several successive passages were done to achieve the yeast form. The morphology of the yeast cells was examined on wet mounts with 85% lactic acid.

Nucleotide sequence accession numbers. The newly reported sequences generated in this study were deposited in the GenBank/EMBL/DDBJ database under the accession numbers listed in Table 1.

RESULTS

Phylogeny. With the primers used, we were able to amplify and sequence 685 bp of CAL loci. Parsimony analysis of the CAL data set yielded 5,000 trees with 278 steps in length in which 20 nodes received 100% bootstrap support. All trees had a consistency index of 0.8885, a retention index of 0.9869, and a homoplasy index of 0.1115. There were 474 constant, 180 parsimony-informative, and 34 variable parsimony-uninformative characters in this fragment, which resulted in a total of 45 haplotypes. One of the most parsimonious trees is shown in Fig. 1. The isolates were distributed in five main clades (clades I to V) that were 100% statistically supported. All isolates of clinical origin were included in the first three clades (clades I to III), which correspond to the same main lineages obtained in our previous study (20). Clade I grouped only Brazilian isolates. Clade II included practically all of the American isolates. As in our previous study (20), clade II was again divided into two highly supported subclades (subclades IIa and IIb). The former subclade grouped most of the isolates from the United States and South America (Argentina, Bolivia, Colombia, Peru, and Venezuela), the type strain of *S. schenckii*, and the type strain of *Dolichoascus schenckii*, while subclade IIb included the rest of isolates from South America (Peru and Argentina) and the only existing isolate from South Africa. Clade III was comprised of isolates from China, India, Italy, Japan, Spain, and the United States and the type strain of *Sporotrichum tropicale*, which was from England. Four environmental isolates formed the two basal clades, clades IV and V. Clade IV grouped two isolates from Mexico, and clade V grouped two isolates identified as being *S. albicans*, one from England (the type strain) and one from Germany. The only existing isolate of *S. schenckii* var. *luriei* and the type strain of *S. inflata* could not be included in the phylogenetic tree because of sequences that were shorter (ca. 300 bp) than the rest of the isolates due to the low specificity of the degenerate primer CL1. However, the length of these sequences was informative enough to prove that both taxa are considerably genetically distant from the rest of isolates included in this study.

Physiologic studies. All isolates demonstrated optimal growth between 20 and 30°C, and none grew at 40°C. The isolates in clades IV and V grew more quickly at 20 and at 30°C, and those in clade I showed the slowest growth at these same temperatures. All the isolates grew well at 35°C; however, those in clades III and IV showed the most restricted growth. Most isolates also grew at 37°C, except for the majority of strains in clade III. The mean colony diameters for isolates in the different clades tested at various temperatures are summarized in Table 2.

A relatively large number of carbon and nitrogen sources were assimilated by all the isolates tested, with no significant differences among them (data not shown). The most important variations were observed in the assimilation of sucrose, raffinose, and ribitol (Table 3). All the isolates were able to split urea after 8 days of incubation, none produced extracellular polysaccharides, and variable results were obtained for acid and gelatinase production. All the isolates tolerated cycloheximide at 0.25% and 10% $MgCl_2$. Variable results were obtained for tolerance to different concentrations of NaCl among isolates of the same clade (data not shown).

Most isolates were able to convert, at least partially, to a yeast phase, and no significant differences were observed among clades.

Morphological studies. The macroscopic morphologies of all isolates were similar regardless of the medium on which they were grown. After 21 days of incubation, colonies on PDA were pale orange to gray-orange, and on CMA and oatmeal agar, they were brown to dark brown. An exception was noted in clade V and in two isolates of the clade IIa (CBS 359.36, the type strain of *S. schenckii*, and NBRC 8158), where colonies remained colorless. Since all the isolates sporulated considerably better on CMA than on other media tested and no growth differences were observed at 25 and 30°C, this culture medium, incubated at 30°C, was used to compare microscopic features among clades. Practically all the isolates developed terminal or intercalary conidial clusters on more or less differentiated conidiophores. The conidia were produced sympodially on denticulate conidiogenous cells. These conidia were hyaline or slightly pigmented, usually obovoid or pear-shaped, and mea-

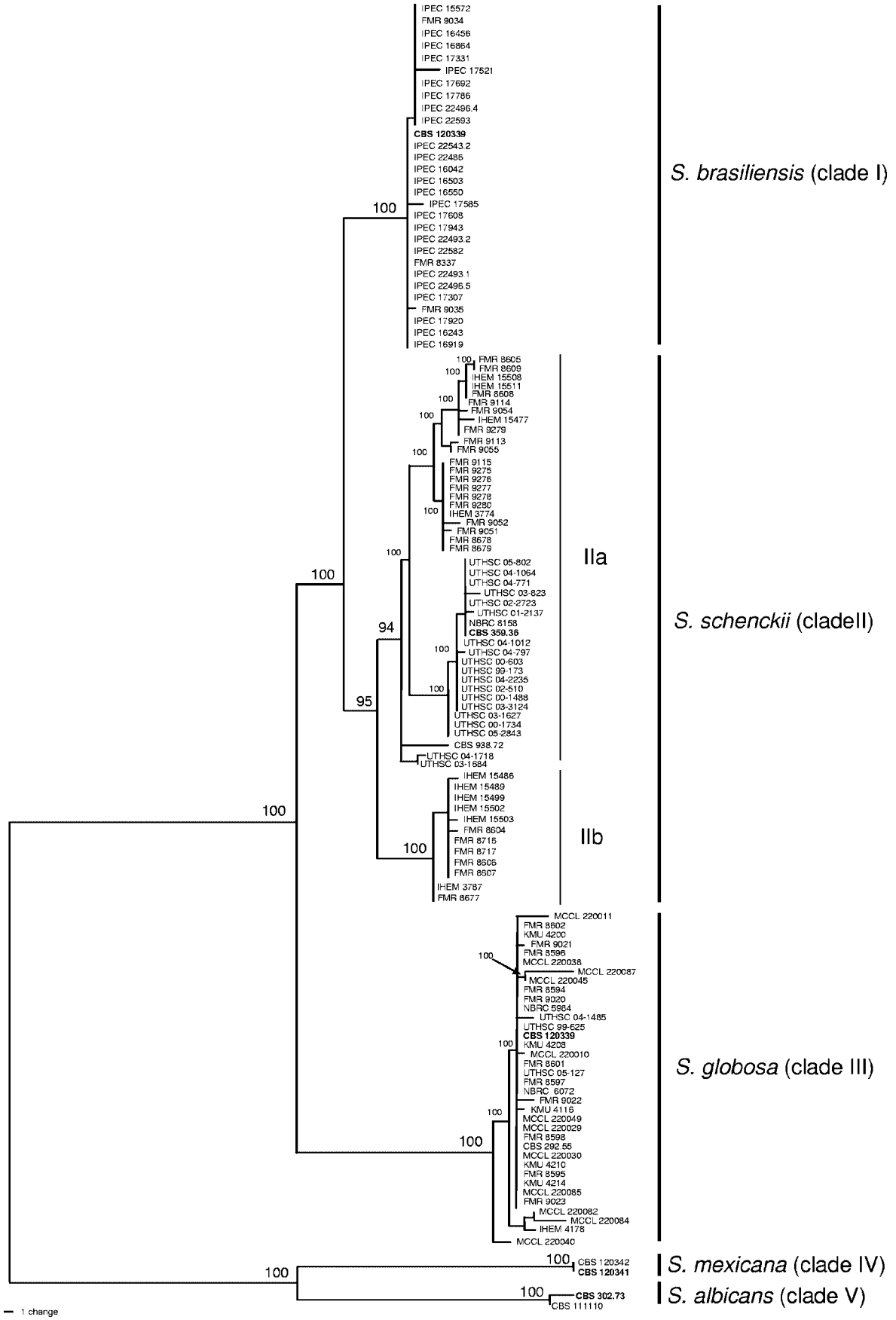

FIG. 1. One of the 5,000 most parsimonious trees obtained from heuristic searches based on analysis produced from the combined data set. Bootstrap support values above 90% are indicated at the nodes. Type strains are indicated with boldface type.

TABLE 2. Mean colony diameter of isolates of the *S. schenckii* complex on PDA at 21 days

Clade	Species	Mean colony diam (mm) ± SD at temp:			
		20°C	30°C	35°C	37°C
I	*S. brasiliensis*	24.7 ± 6.1	24.7 ± 5.5	16.6 ± 5.9	7.6 ± 1.6
IIa	*S. schenckii*	28.9 ± 5.4	36.0 ± 5.5	21.3 ± 7.0	6.1 ± 3.0
IIb	*S. schenckii*	30.1 ± 3.0	33.6 ± 3.0	20.8 ± 3.5	5.9 ± 1.6
III	*S. globosa*	28.5 ± 7.0	30.9 ± 5.0	11.5 ± 5.3	0.4 ± 0.5
IV	*S. mexicana*	53.5 ± 0.7	67.5 ± 2.1	10.8 ± 0.4	1.8 ± 0.4
V	*S. albicans*	51.0 ± 2.8	67.0 ± 4.2	28.0 ± 1.4	4.2 ± 1.4

sured 2 to 8 μm long by 1.5 to 2.5 μm wide. In addition, practically all the isolates produced another type of conidia, which were thick walled, dark brown, and usually borne individually on short denticles along the sides of the vegetative hyphae. These conidia, which are regarded as sessile conidia, measured 2 to 6 μm long by 2 to 3.5 μm wide and showed different shapes among clades. The presence or absence of these sessile conidia and their morphologies were key features to distinguish the different clades obtained in the phylogenetic analysis. Clades I and III showed globose to subglobose sessile conidia (Fig. 2A and D). Most of the isolates of clade II produced, in greater or lesser degree, triangular to cuneiform sessile conidia (Fig. 2 B). Only two isolates in this clade (FMR 8677 and IHEM 3787), which were phylogenetically distinct from the rest of the isolates, showed a different type of sessile conidia, which were obovoid, elongated, or irregularly shaped (Fig. 2C). Isolates of clade IV produced subglobose, obovoidal, or ellipsoidal sessile conidia (Fig. 2E). Neither the isolates in clade V nor the two isolates in clade IIa produced pigmented sessile conidia. These conidial differences correlated with those observed for growth rate and carbohydrate assimilations mentioned above (Tables 2 and 3) and confirmed that clades I, III, IV, and V represent species that are different from *S. schenckii*. The first three clades are here proposed as new species, and clade V corresponded with *S. albicans*.

Sporothrix brasiliensis Marimon, Gené, Cano, et Guarro, sp. nov. = *Sporothrix schenckii*, clade I sensu Marimon et al. (20). Coloniae in PDA ad 30°C post 21 dies 15 vel 38 mm diametri. Augmentum fit in temperatura 37°C. Conidia sympodialia hyalina vel subhyalina, obovoidea, 2 vel 6 per 1 vel 4 μm. Conidia sessilis brunnea vel atrobrunnea, crassitunicata, plerumque globosa vel subglobosa, 2.5 vel 5 per 2 vel 3 μm. Teleomorphosis ignota. Assimilantur ribitolum variabile. Non assimilantur sucrosum et raffinosum.

The colonies on PDA attained a diameter of 15 to 38 mm after 21 days of incubation at 30°C. Conidiogenous cells were usually terminal or intercalary on more or less differentiated conidiophores, were slightly swollen, and produced conidia sympodially on a few denticles. Sympodial conidia were usually hyaline to subhyaline, obovoidal, and 2 to 6 μm long by 1 to 4 μm wide. Sessile conidia were brown to dark brown, thick walled, globose to subglobose, and 2.5 to 5 μm long by 2 to 3 μm wide. A teleomorph was not developed by any isolate. The maximum growth temperature was 37°C (5 to 10 mm in diameter after 21 days). The fungus did not grow at 40°C and was unable to assimilate sucrose and raffinose. Variable results were seen for the assimilation of ribitol (81.5% of isolates were negative). The holotype is IMI 394469, from Rio de Janeiro, Brazil. Ex-type living cultures include CBS 120339, FMR 8309, and IPEC 16490. Etymology refers to the country of origin of the isolates.

TABLE 3. Physiological key characteristics for differentiating the clades of the *S. schenckii* complex

Clade	Species	% of isolates that assimilate carbon source:		
		Sucrose	Raffinose	Ribitol
I	*S. brasiliensis*	0	0	18.5
IIa	*S. schenckii*	100	100	100
IIb	*S. schenckii*	100	100	33.3
III	*S. globosa*	100	0	90.9
IV	*S. mexicana*	100	100	100
V	*S. albicans*	100	0	50

Sporothrix globosa Marimon, Gené, Cano, et Guarro, sp. nov. = *Sporothrix schenckii*, clade III sensu Marimon et al. (20). Coloniae in PDA ad 30°C post 21 dies 18 vel 40 mm diametri. Augmentum fit in temperatura 35°C. Conidia sympodialia hyalina vel subhyalina, obovoidea, 2.5 vel 5 per 1 vel 3 μm. Conidia sessilis brunnea vel atrobrunnea, crassitunicata, plerumque globosa vel subglobosa, 3 vel 4 per 2 vel 3.5 μm. Teleomorphosis ignota. Assimilantur sucrosum et ribitolum. Non assimilantur raffinosum.

The colonies on PDA attained a diameter of 18 to 40 mm after 21 days of incubation at 30°C. Conidiogenous cells were often terminal or intercalary on more or less differentiated conidiophores, were often swollen, and produced conidia sympodially on numerous denticles. Sympodial conidia were usually hyaline to subhyaline, obovoidal, and 2 to 5 μm long by 1 to 3 μm wide. Sessile conidia were brown to dark brown, thick walled, predominantly globose to subglobose, and 2.5 to 4 μm long by 2 to 3.5 μm wide. A teleomorph was not developed by any isolate. The maximum growth temperature was 35°C (2.5 to 20 mm in diameter after 21 days). All isolates were unable to grow at 37°C, with the exception of four strains, which showed a very restricted growth (up to 2 mm in diameter after 21 days). The fungus did not grow at 40°C and was unable to assimilate raffinose. Ribitol was assimilated by 90.9% of the isolates. The holotype is IMI 394470 from Zaragoza, Spain. Ex-type living cultures include CBS 120340 and FMR 8600. Etymology refers to the spherical shape of the lateral conidia.

Sporothrix mexicana Marimon, Gené, Cano, et Guarro, sp. nov. = *Sporothrix schenckii*, clade IV, from the present study. Coloniae in PDA ad 30°C post 21 dies 66 vel 69 mm diametri. Augmentum fit in temperatura 37°C. Conidia sympodialia hyalina vel subhyalina, obovoidea, 3 vel 5.5 per 2 vel

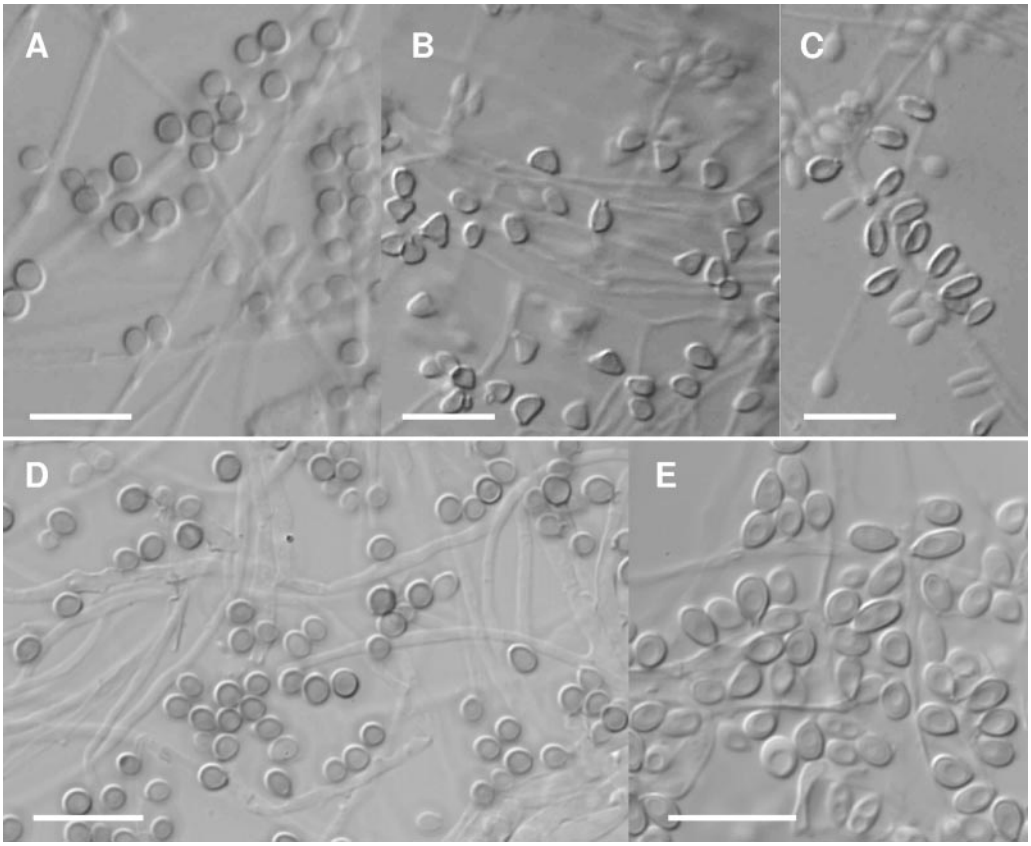

FIG. 2. Morphology of the sessile conidia of the *S. schenckii* species complex. (A) *S. brasiliensis* CBS 120339 (clade I). (B and C) *S. schenckii* (clade II) and FMR 8608 (clade IIa) (B) and FMR 8677 (clade IIb) (C). (D) *S. globosa* CBS 120340 (clade III). (E) *S. mexicana* CBS 120341 (clade IV). Bars, 10 μm.

2.5 μm. Conidia sessilis brunnea vel atrobrunnea, crassitunicata, subglobosa, obovoides vel ellipsoidea, 3 vel 4 per 2 vel 3.5 μm. Teleomorphosis ignota. Assimilantur ribitolum, sucrosum, et raffinosum.

The colonies on PDA attained a diameter of 66 to 69 mm after 21 days of incubation at 30°C. Conidiogenous cells were usually terminal or intercalary on more or less differentiated conidiophores, were often swollen, and were densely denticulate. Sympodial conidia were usually hyaline to subhyaline, obovoidal, and 3 to 5.5 μm long by 2 to 2.5 μm wide. Sessile conidia were brown to dark brown, thick walled, predominantly subglobose, obovoidal, or ellipsoidal, and 3 to 4 μm long by 2 to 3.5 μm wide. A teleomorph was not developed by any isolate. The maximum growth temperature was 37°C (1.5 to 2.5 mm in diameter after 21 days). The fungus did not grow at 40°C and was able to assimilate sucrose, raffinose, and ribitol. The holotype is IMI 394471 from Puebla, Mexico. Ex-type living cultures include CBS 120341 and FMR 9108. Etymology refers to the country of origin of the isolates.

DISCUSSION

Up to now, sporothrichosis, the most common subcutaneous fungal infection in South America, had been attributed to a unique species, *S. schenckii*. However, a recent molecular study demonstrated that *S. schenckii* is a complex of at least six putative phylogenetic species (20). Indeed, something similar has occurred in many other pathogenic fungi where the use of different molecular markers has demonstrated that they are genetically more complex than was initially thought. This is the case for *Pneumocystis carinii* (1), *Coccidioides immitis* (17), *Aspergillus fumigatus* (26), *Candida parapsilosis* (29), and *Pseudallescheria boydii* (10), among others. All of them constitute species complexes that are often difficult to differentiate phenotypically but sometimes with different clinical manifestations and infecting different body sites or even different hosts.

The most useful and significant finding of the present study has been the demonstration of a clear correlation between molecular data and phenotypic features, which allowed us to differentiate three new *Sporothrix* species, two of which have been associated with human infections (*S. brasiliensis* and *S. globosa*). Another interesting aspect of this study has been to confirm that the CAL gene is a good marker for the recognition of these species. Thus, by sequencing only this one locus and including many additional strains in the analysis, we were able to obtain the same main groups as in the previous study, where we sequenced three different loci (20). In addition, the analysis of the CAL sequences was useful to demonstrate that *S. albicans*, *S. inflata*, and *S. schenckii* var. *luriei* are clearly different species from *S. schenckii*.

In order to confirm the uniqueness of the new species described here, and knowing that several ascomycetes belonging

mainly to *Ophiostoma* species can form *Sporothrix* anamorphs and that β-tubulin sequences of several of these ascomycetes are deposited in the GenBank database, we sequenced a representative isolate of each of the new species (*S. brasiliensis* AM116946, *S. globosa* AF116966, and *S. mexicana* AM498344) for comparison. In all cases, the degree of homology with the deposited sequences was very low.

Our study, to some extent, has confirmed previous molecular studies carried out by other authors, who already demonstrated high genetic variability in *S. schenckii* (13, 14, 15, 22, 32). Watanabe et al. (32), using restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer region, grouped 204 isolates into four types, which correlated with their geographical origins. Type I was found predominantly in Africa and America, type II was found predominantly in South America, type III was found predominantly in North America, and type IV was found predominantly in Australia and Asia. This geographical distribution is similar to that obtained here. However, in our case, the Asian isolates grouped with the European ones, forming a highly supported group, which was the basis of the newly proposed species *S. globosa*. This agrees with data from a study reported previously by Ishizaki et al. (14), who used RFLP analysis of mitochondrial DNA, where the European isolates (all from Spain) were nested in the same phylogenetic group as isolates from Korea, China, and Japan. In the *S. globosa* clade, five Chinese environmental isolates were included which had previously been identified as being *S. schenckii* isolates by mitochondrial DNA RFLP by Ishizaky et al. (15). These isolates showed the same key phenotypic features as the rest of the isolates in the clade. With the exception of the Brazilian isolates and three isolates from the United States, the rest of the American isolates were included in clade II, as in the previous study (20). This clade was the most genetically heterogeneous group and probably encompasses a few more putative phylogenetic species. However, we did not find enough phenotypic characteristics to distinguish them. This clade also included the type strain of *S. schenckii*, which was also of American origin.

The saprophytic form of *S. schenckii* is usually characterized by the two types of conidia (5, 19) described above, i.e., sessile and sympodial. While the shape of the former type of conidia is very variable (globose, subglobose, ellipsoidal, triangular, etc.) among isolates, the morphology of the latter is more constant, being more or less obovoidal in general. By examining the morphology of the sessile conidia, we found robust differences among clades, which, combined with physiological data, allowed the differentiation of some cryptic species within the set of isolates that we received as *S. schenckii*. Other authors also correlated the morphology of the sessile conidia with some physiological features when environmental isolates of *S. schenckii* were studied. For instance, strains reported previously by Mackinnon (18), which were isolated from different environmental sources, showed oval dark-pigmented conidia and multiple spicules on the hyphae after conidial detachment. These isolates grew at 37°C and were pathogenic to mice. By contrast, other environmental strains isolated previously by Howard and Orr (12) formed dark-pigmented conidia firmly attached to the hyphae. These isolates did not grow at 37°C and were nonpathogenic to mice. Mesa-Arango et al. (22) found significant differences in conidial size among several groups generated by random amplified polymorphic DNA analysis, which coincided with different geographical locations. However, they did not indicate to what type of conidia they referred, and it is important to take into account that, in general, the sporulation of *Sporothrix* spp., especially the production of the sessile pigmented conidia, can vary considerably when routine culture media such as Sabouraud dextrose agar or PDA are used. We recommend the use of CMA for determining the microscopic features of these fungi, as the morphology remains stable when this medium is used. We were surprised by the fact that the type strain of *S. schenckii* (CBS 359.36) produced only hyaline sympodial conidia. This strain has traditionally been described as having pigmented conidia (11); however, we were unable to observe any dark, sessile conidia, and we presume that the isolate has degenerated, thereby losing its ability to produce these conidia. Isolate NBRC 8158, which is morphologically and genetically identical to the above-described isolate and of unknown origin, is probably a subculture of the type strain.

Only a few studies on the physiology of *S. schenckii* have been published. Ghosh et al. (9) previously tested 49 isolates from India, and none of them tolerated a 10% salt concentration. In contrast, approximately one-half of the isolates in our study tolerated this concentration, although isolates of *S. brasiliensis* showed the most restricted growth under these conditions. We also noticed an important discrepancy with the results reported by Ghosh et al. (9) concerning the carbohydrate assimilation tests, specifically inositol and mannitol. Both tests were negative for all the isolates tested previously by Ghosh et al. (9), while in our study, they were consistently positive. The results of assimilation studies for cellobiose, ribitol, D-galactose, D-glucose, glycerol, maltose, sorbitol, trehalose, and D-xylose were similar in both studies. Ghosh et al. (9) found variable results for the assimilation of raffinose, rhamnose, and dextrin within a given geographical area. We also found variable results for raffinose; however, all our isolates were able to assimilate rhamnose and dextrin. One explanation for these important discrepancies could lie in the fact that Ghosh et al. (9) used an auxanographic method with discs impregnated with saturated solutions of the carbohydrates on solid medium incubated at 37°C, while we used liquid media incorporated into microplates and incubated at 25°C. Although we evaluated the responses to more than 40 physiological tests, we found that only the assimilation of sucrose, raffinose, and ribitol was useful in discriminating members of the *S. schenckii* complex. Our studies are in agreement with those described previously by Dixon et al. (8) with regard to the ability of environmental isolates to grow at 35°C. The growth of all clinical isolates at 37°C was not, however, supported by our findings, as *S. globosa* failed to grow at this temperature.

In conclusion, *S. schenckii* must no be longer considered a single species. Using the procedures described here and the data reported in Table 4, the different species within the complex can be easily and reliably identified without the need for molecular techniques. As more epidemiological data become available for these species, we should gain a clear understanding of their geographic distribution, their role in disease, and the potentially different responses to antifungal agents.

TABLE 4. Summary of the key features for species differentiation

Species	Presence of sessile pigmented conidia	Colonies on PDA at 30°C exceeding 50 mm in 21 days	Growth at 37°C	Assimilation test result	
				Sucrose	Raffinose
S. albicans	No	Yes	Yes	+	−
S. brasiliensis	Yes	No	Yes	−	−
S. globosa	Yes	No	No	+	−
S. mexicana	Yes	Yes	Yes	+	+
S. schenckii	Yes	No	Yes	+	+

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